METHODS AND COMPOSITIONS FOR DETERMINATION OF GLYCATED PROTEINS

BACKGROUND OF THE INVENTION

[0001] A glycated protein is a substance which is produced by the non-enzymatic and irreversible binding of the amino group of an amino acid constituting a protein, with the aldehyde group of a reducing sugar such as aldose. *See e.g.*, U.S. Patent No. 6,127,138. Such a non-enzymatic and irreversible binding reaction is also called "Amadori rearrangement," and therefore the above-mentioned glycated protein may also be called "Amadori compound" in some cases.

[0002] Nonenzymatic glycation of proteins has been implicated in the development of certain diseases, *e.g.*, diabetic complications and the aging process (Takahashi et al., *J. Biol. Chem.*, 272(19):12505-7 (1997); and Baynes and Monnier, *Prog. Clin. Biol. Res.*, 304:1-410 (1989)). This reaction leads to dysfunction of target molecules through formation of sugar adducts and cross-links. Considerable interest has focused on the Amadori product that is the most important "early" modification during nonenzymatic glycation *in vitro* and *in vivo*.

[0003] Various assays for glycated proteins are known. For example, U.S. Patent No. 6,127,138 discloses that a sample containing a glycated protein is treated with Protease XIV or a protease from *Aspergillus genus*, thereafter (or while treating the sample with the above protease) FAOD (fructosyl amino acid oxidase) is caused to react with the sample so as to measure the amount of oxygen consumed by the FAOD reaction or the amount of the resultant reaction product, thereby to measure the glycated protein.

[0004] In another example, U.S. Patent No. 6,008,006 discloses that the amount of glycated proteins in a sample can be quantified by reacting the sample with first a reagent which is a combination of a protease and a peroxidase and second with a ketoamine oxidase. U.S. Patent No. 6,008,006 also discloses a kit

which contains the combined peroxidase/protease enzyme reagent and also the ketoamine oxidase.

BRIEF SUMMARY OF THE INVENTION

[0005] In one aspect, the present invention is directed to an isolated chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase.

[0006] In another aspect, the present invention is directed to an isolated nucleic acid comprising a nucleotide sequence encoding a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase. Recombinant cells comprising the nucleic acid and methods for producing the chimeric protein using the nucleic acid are also provided.

[0007] In still another aspect, the present invention is directed to a method for assaying for a glycated protein in a sample, which method comprises: a) contacting a sample to be assayed with a protease to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) contacting said generated glycated peptide or glycated amino acid with a chimeric protein comprising, from N-terminus to C-terminus: i) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and ii) a second peptidyl fragment comprising an amadoriase, to oxidize said glycated peptide or glycated amino acid; and c) assessing oxidation of said glycated peptide or glycated amino acid by said chimeric protein to determine the presence and/or amount of said glycated protein in said sample.

[0008] In yet another aspect, the present invention is directed to a kit for assaying for a glycated protein in a sample, which kit comprises: a) a protease to generate glycated peptide or glycated amino acid from a glycated protein, if contained in a sample; b) the above-described chimeric protein to oxidize said glycated peptide or glycated amino acid; and c) means for assessing oxidation of

said glycated peptide or glycated amino acid by said chimeric protein to determine the presence and/or amount of said glycated protein in said sample.

[0009] In yet another aspect, the present invention is directed to a method for assaying for a glycated protein in a sample, which method comprises: a) contacting a sample to be assayed with a proteinase K to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) contacting said generated glycated peptide or glycated amino acid with an amadoriase to oxidize said glycated peptide or glycated amino acid; and c) assessing oxidation of said glycated peptide or glycated amino acid by said amadoriase to determine the presence and/or amount of said glycated protein in said sample.

[0010] In yet another aspect, the present invention is directed to a kit for assaying for a glycated protein in a sample, which kit comprises: a) a proteinase K to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) an amadoriase to oxidize said glycated peptide or glycated amino acid; and c) means for assessing oxidation of said glycated peptide or glycated amino acid by said amadoriase to determine the presence and/or amount of said glycated protein in said sample.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0011] Figure 1 illustrates a comparison between an exemplary GSP kit and a Randox Fructosamine kit.

[0012] Figure 2 illustrates assay linearity of an exemplary method for assaying for a glycated protein in a sample.

[0013] Figures 3 and 4 show the dose-dependent reaction with fructosyl-valine.

[0014] Figure 5 shows the dose-dependent signal with patient hemoglobin digested with a proteinase (5 min. digestion).

DETAILED DESCRIPTION OF THE INVENTION

[0015] For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

A. Definitions

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

[0017] As used herein, "a" or "an" means "at least one" or "one or more."

[0018] As used herein, a "leader sequence" refers to a peptide sequence, when fused to a target peptide or protein, increases stability and/or expression level of the target peptide or protein. Normally, a leader sequence increases stability and/or expression level of the target peptide or protein for at least 50%. Preferably, a leader sequence increases stability and/or expression level of the target peptide or protein for at least 1 fold, 2 folds, 5 folds, 10 folds or more than 10 folds. In the regulation of gene expression for enzymes concerned with amino acid synthesis in prokaryotes, the leader sequence codes for the leader peptide that contains several residues of the amino acid being regulated. Transcription is closely linked to translation, and if translation is retarded by limited supply of aminoacyl tRNA for the specific amino acid, the mode of transcription of the leader sequence permits full transcription of the operon genes; otherwise complete transcription of the leader sequence prematurely terminates transcription of the regulated gene.

[0019] As used herein, a "glycated protein" refers to a substance which is produced by the non-enzymatic and irreversible binding of the amino group of an amino acid constituting a protein, with the aldehyde group of a reducing sugar such as aldose. *See e.g.*, U.S. Patent No. 6,127,138. Such a non-enzymatic and irreversible binding reaction is also called "Amadori rearrangement," and

therefore the above-mentioned glycated protein may also be called "Amadori compound" in some cases.

[0020] As used herein, an "amadoriase" refers to an enzyme catalyzing the oxidative deglycation of Amadori products to yield corresponding amino acids, glucosone, and H_2O_2, as shown in the following reaction:

$$R_1\text{-}CO\text{-}CH_2\text{-}NH\text{-}R_2 + O_2 + H_2O \rightarrow R_1\text{-}CO\text{-}CHO + R_2\text{-}NH_2 + H_2O_2$$

wherein, R_1 represents the aldose residue of a reducing sugar and R_2 represents a residue of an amino acid, protein or peptide. Other synonyms of amadoriase include fructosyl amino acid oxidase (FAOD) and fructosyl amine:oxygen oxidoreductase (FAOO). For purposes herein, the name "amadoriase" is used herein, although all such chemical synonyms are contemplated. "Amadoriase" also encompasses a functional fragment or a derivative that still substantially retain its enzymatic activity catalyzing the oxidative deglycation of Amadori products to yield corresponding amino acids, glucosone, and H_2O_2. Typically, a functional fragment or derivative retains at least 50% of its amadoriase activity. Preferably, a functional fragment or derivative retains at least 60%, 70%, 80%, 90%, 95%, 99% or 100% of its amadoriase activity. It is also intended that an amadoriase can include conservative amino acid substitutions that do not substantially alter its activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson, *et al.*, *Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. Co., p. 224). Such exemplary substitutions are preferably made in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	**Conservative substitution**
Ala (A)	Gly; Ser

Original residue	Conservative substitution
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

[0021] As used herein, "glycohemoglobin" refers to a fructosylamine derivative produced by the glycation of hemoglobin in blood.

[0022] As used herein, "glycoalbumin" refers to a fructosylamine derivative produced by the glycation of albumin in blood.

[0023] As used herein, "fructosamine" refers to a derivative (having a reducing ability) produced by the glycation of a protein in blood.

[0024] As used herein, a "composition" refers to any mixture of two or more products or compounds. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous, or any combination thereof.

[0025] As used herein, a "combination" refers to any association between two or among more items.

[0026] As used herein, "plasma" refers to the fluid, noncellular portion of the blood, distinguished from the serum obtained after coagulation.

[0027] As used herein, "serum" refers to the fluid portion of the blood obtained after removal of the fibrin clot and blood cells, distinguished from the plasma in circulating blood.

[0028] As used herein, "fluid" refers to any composition that can flow. Fluids thus encompass compositions that are in the form of semi-solids, pastes, solutions, aqueous mixtures, gels, lotions, creams, and other such compositions.

[0029] As used herein, "peroxidase" refers to an enzyme that catalyses a host of reactions in which hydrogen peroxide is a specific oxidizing agent and a wide range of substrates act as electron donors. It is intended to encompass a peroxidase with conservative amino acid substitutions that do not substantially alter its activity. The chief commercially available peroxidase is horseradish peroxidase.

[0030] As used herein, "glucose oxidase" refers to an enzyme that catalyzes the formation of gluconic acid and H_2O_2 from glucose, H_2O and O_2. It is intended to encompass glucose oxidase with conservative amino acid substitutions that do not substantially alter its activity.

[0031] As used herein, "hexokinase" refers to an enzyme that catalyses the transfer of phosphate from ATP to glucose to form glucose-6-phosphate, the first reaction in the metabolism of glucose via the glycolytic pathway. It is intended to encompass hexokinase with conservative amino acid substitutions that do not substantially alter its activity.

[0032] As used herein, the abbreviations for any protective groups, amino acids and other compounds, are in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature, unless otherwise indicated (*see Biochemistry* 11: 1726 (1972)).

B. Chimeric proteins comprising an amadoriase and nucleic acids encoding the same

[0033] In one aspect, the present invention is directed to an isolated chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase.

[0034] Any suitable bacterial leader sequences can be used. As disclosed in U.S. Patent No. 6,194,200, expression of the polypeptide of interest as a fused protein with a leader sequence from another gene has several advantages in addition to providing for stability. For example, the presence of the N-terminal amino acids provides a means for using general purification techniques for purification of any of a variety of polypeptides. For example, the N-terminal amino acids of the N-protein are predictably antigenic, and thus specific antibodies raised against the N-terminal amino acids of the N-protein may be used for the amino purification of the fusion proteins containing the N-terminus of the N-protein. Furthermore, the N-terminus of the N-protein has a high positive charge, which facilitates purification of the desired protein by ion-exchange chromatography, and the like.

[0035] The leader sequence can also be a hydrophobic amino acid sequence, which may additionally function as a signal sequence for secretion. *See* U.S. Patent No. 6,194,200. A DNA sequence encoding the signal sequence is joined upstream from and in reading frame with the gene of interest. Typically, the signal sequence includes a cleavage site which is recognized by a signal sequence peptidase. Thus, positioning the polypeptide of interest directly after the signal sequence cleavage site will allow it to be specifically cleaved from the signal sequence and secreted as a mature polypeptide. Examples of hydrophobic amino acid sequences include the bacterial alkaline phosphatase signal sequence; the OMP-A, B, C, D, E or F signal sequences; the LPP signal sequence, β-lactamase signal sequence; and toxin signal sequences.

[0036] Other leader sequences which can be used include hydrophilic sequences, for example the N-terminal 41 amino acid residues from amphiregulin which may provide for modification of the function of the polypeptide of interest. *See* U.S. Patent No. 6,194,200. In addition, a cytotoxic agent such as a toxin A-chain fragment, ricin A-chain, snake venom growth arresting peptide, or a targeting molecule such as a hormone or antibody can be coupled covalently with the leader sequence with in most cases minimal effect on the biological activity of the gene product of interest. As with the other leader sequences, a DNA sequence

encoding the leader sequence is joined upstream from and in reading frame with the gene of interest.

[0037] Where the leader sequence is not a signal sequence or does not contain a convenient natural cleavage site, additional amino acids may be inserted between the gene of interest and the leader sequence to provide an enzymatic or chemical cleavage site for cleavage of the leader peptide, following purification of the fusion protein, to allow for subsequent purification of the mature polypeptide. *See* U.S. Patent No. 6,194,200. For example, introduction of acid-labile aspartyl-proline linkages between the two segments of the fusion protein facilitates their separation at low pH. This method is not suitable if the desired polypeptide is acid-labile. The fusion protein may be cleaved with, for example, cyanogen bromide, which is specific for the carboxy side of methionine residues. Positioning a methionine between the leader sequence and the desired polypeptide would allow for release of the desired polypeptide. This method is not suitable when the desired polypeptide contains methionine residues.

[0038] Other bacterial leader sequences disclosed in the following patents, patent application and references can also be used: WO 00/28041 and WO 89/03886; U.S. Patent Nos. 5914250, 5885811, 5171670, 5030563, 4948729 and 4588684; EP Patent Nos. EP 0,196,864, EP 0,186,643 and EP 0,121,352; Michiels et al., *Trends Microbiol.*, 9(4):164-8 (2001); Hobom et al., *Dev. Biol. Stand.*, 84:255-62 (1995); Hardy and Randall, *J. Cell. Sci. Suppl.*, 11:29-43 (1989); Saier et al., *FASEB J.*, 2(3):199-208 (1988); and Peakman et al., *Nucleic Acids Res.*, 20(22):6111-2 (1992). Preferably, the bacterial leader sequence is a leader sequence of an *E.coli.* protein, *e.g.*, the *E.coli.* leader sequences disclosed in Roesser and Yanofsky, *Nucleic Acids Res.*, 19(4):795-800 (1991); and Kuhn et al., *Mol. Gen. Genet.*, 167(3):235-41 (1979).

[0039] In one example, the leader sequence has at least 40% identity to the amino acid sequence set forth in SEQ ID NO:1 (MGGSGDDDDLAL), in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:1. Preferably, the leader sequence has at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% identity to

the amino acid sequence set forth in SEQ ID NO:1, in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:1. Also preferably, the leader sequence binds to an antibody that specifically binds to an amino acid sequence set forth in SEQ ID NO:1. Still preferably, the leader sequence comprises the amino acid sequence set forth in SEQ ID NO:1.

[0040] The first peptidyl fragment can have any suitable length. For example, the first peptidyl fragment comprises about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, ,26, 27, 28, 29 or 30 amino acid residues. Preferably, the first peptidyl fragment comprises about 20 amino acid residues.

[0041] Any suitable amadoriase can be used. In one example, the amadoriase is of *Aspergillus sp.* origin (*See e.g.*, Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997)). In another example, the amadoriase uses FAD as a cofactor. Preferably, the amadoriase has a FAD cofactor-binding consensus sequence Gly-X-Gly-X-X-Gly (SEQ ID NO:2), X being any amino acid residue. In still another example, the amadoriase is amadoriase Ia, amadoriase Ib, amadoriase Ic or amadoriase II (*See e.g.*, Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997)). Amino acid sequence homology between the N-terminal sequence of amadoriases Ia, amadoriase Ib, amadoriase Ic and amadoriase II is shown in the following Table 2. These data were obtained by a computerized search using the combined GenBank™ CDS translations/PDB/SwissProt/SPupdate/PIR data base (*See* Table IV of Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997)). Numbers indicate the amino acid positions within each sequence. Conservative substitutions are indicated by (+). The data of amadoriases correspond to the N-terminal sequences obtained in Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997).

Table 2. Amino acid sequence homology between the N-terminal sequence of amadoriase Ia, amadoriase Ib, amadoriase Ic and amadoriase II

Protein	Sequences with residue	SEQ ID NO

	nos.	
Amadoriases Ia	^{1}A P S I L S T E S S I (C/T) V I G A G T W G^{20}	SEQ ID NO:7
Amadoriase Ib	^{1}A P S I L S T E S S I I V I G A G T W G^{20}	SEQ ID NO:8
Amadoriase Ic	^{1}S T E S S I I V I G A G T W G (C) (S)T A L^{20}	SEQ ID NO:9
Amadoriase II	^{1}A V T K S S S L L I V G A G T W G T S T^{20}	SEQ ID NO:10

[0042] Other amadoriases, *e.g.*, amadoriases disclosed in GenBank Accession No. U82830 (Takahashi et al., *J. Biol. Chem.*, 272(19):12505-12507 (1997) and amadoriases disclosed U.S. Patent No. 6,127,138 can also be used. A functional fragment or a derivative of an amadoriase that still substantially retain its enzymatic activity catalyzing the oxidative deglycation of Amadori products to yield corresponding amino acids, glucosone, and H_2O_2 can also be used.

[0043] Normally, a functional fragment or a derivative of an amadoriase retain at least 50% of its enzymatic activity. Preferably, a functional fragment or a derivative of an amadoriase retain at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% of its enzymatic activity.

[0044] Assays for enzymatic activities of amadoriases are known in the art (*See e.g.*, Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997) and U.S. Patent No. 6,127,138). Four exemplary assays for enzymatic activities of amadoriases are disclosed in Takahashi et al., *J. Biol. Chem.*, 272(6):3437-43 (1997).

Glucosone Formation

[0045] In this assay, the enzyme activity is monitored by the release of glucosone measured by a colorimetric reaction with OPD using fructosyl propylamine as a substrate. This assay is based on the end point measurement of

glucosone formed after 120 min of reaction time. The reaction mixture contains 20 mM sodium phosphate, pH 7.4, 10 mM OPD, 10 mM fructosyl propylamine, and enzyme protein in a final volume of 1 ml. After incubation at 37°C for 2 h, the absorbance at 320 nm is measured. The reaction is linear to 240 min in a dose-dependent manner under these conditions. One unit of enzyme activity is defined as the amount of the enzyme that produces 1 μmol of glucosone/min. Synthesized glucosone is used as a standard.

Free Amine Assay

[0046] To assay the release of free amine, fluorescence is measured after reaction with fluorescamine. Twenty-five (25) μl of a solution of pure enzyme or enzyme-rich fraction, 15 μl of 20% fructosyl propylamine in water, and 250 μl of PBS are incubated at 37°C for different times as indicated. The reaction is stopped by filtration through a Microcon-10 (Amicon, Beverly, MA) at 4 C. One (1) μl of the pure or 1:10 diluted filtrate is added to 1.5 ml of 50 mM phosphate buffer pH 8.0. Under vigorous vortexing 0.5 ml of 0.03% fluorescamine in dioxane is rapidly added. After 5 min fluorescence is measured (λ_{ex} = 390 nm, λ_{em} = 475 nm). A standard plot is made with 6-150 ng of propylamine.

H_2O_2 Assay

[0047] Hydrogen peroxide is quantitated by the quinone dye assay according to Sakai et al., *Biosci. Biotech. Biochem.*, 59:487-491 (1995). The reaction mixture contains 20 mM Tris-HCl, pH 8.0, 1.5 mM 4-aminoantipyrine, 2.0 mM phenol, 2.0 units of peroxidase, 10 mM fructosyl propylamine, and enzyme protein in a total volume of 1 ml. Production of the H_2O_2 is monitored by the formation of a quinone dye following the absorbance at 505 nm ($\varepsilon = 5.13 \times 10^3$). The production of 0.5 μmol of quinone dye corresponds to the formation of 1.0 μmol of H_2O_2.

Oxygen Consumption

[0048] Oxygen consumption is determined with a YSI-Beckman glucometer II equipped with a Clarke type oxygen electrode as described in Gerhardinger, et al., *J. Biol. Chem.*, 270:218-224 (1995). Briefly, enzyme (50 µl) is added to the chamber containing 750 µl of PBS and 650 µl of water. The reaction is started by addition of 50 µl of 300 mM fructosyl propylamine (final concentration 10 mM).

[0049] In another example, the amadoriase has at least 40% identity to the amino acid sequence set forth in SEQ ID NO:3 (AVTKSSSLLIVGAGTWGTSTALHLARRGYTNVTVLDPYPVPSAISAGNDV NKVISSGQYSNNKDEIEVNEILAEEAFNGWKNDPLFKPYYHDTGLLMSAC SQEGLDRLGVRVRPGEDPNLVELTRPEQFRKLAPEGVLQGDFPGWKGYF ARSGAGWAHARNALVAAAREAQRMGVKFVTGTPQGRVVTLIFENNDVK GAVTGDGKIWRAERTFLCAGASAGQFLDFKNQLRPTAWTLVHIALKPEE RALYKNIPVIFNIERGFFFEPDEERGEIKICDEHPGYTNMVQSADGTMMSIP FEKTQIPKEAETRVRALLKETMPQLADRPFSFARICWCADTANREFLIDRH PQYHSLVLGCGASGRGFKYLPSIGNLIVDAMEGKVPQKIHELIKWNPDIAA NRNWRDTLGRFGGPNRVMDFHDVKEWTNVQYRDISKL), in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:3. Preferably, the amadoriase has at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% identity to the amino acid sequence set forth in SEQ ID NO:3, in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:3. Also preferably, the amadoriase binds to an antibody that specifically binds to an amino acid sequence set forth in SEQ ID NO:3. Also preferably, the amadoriase comprises the amino acid sequence set forth in SEQ ID NO:3.

[0050] The first and second peptidyl fragments can be linked via any suitable linkage. For example, the first and second peptidyl fragments can be linked via a cleavable linkage.

[0051] The isolated chimeric protein can further comprise, at its C-terminus, a third peptidyl fragment comprising a second bacterial leader sequence from about 5 to about 30 amino acid residues. Any suitable bacterial leader sequences, including the ones described above, can be used.

[0052] In one example, the second bacterial leader sequence is a leader sequence of an *E.coli.* protein. in another example, the second bacterial leader sequence has at least 40% identity to the amino acid sequence set forth in SEQ ID NO:4 (KGELEGLPIPNPLLRTG), in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:4. Preferably, the second bacterial leader sequence has at least 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% identity to the amino acid sequence set forth in SEQ ID NO:4, in which the percentage identity is determined over an amino acid sequence of identical size to the amino acid sequence set forth in SEQ ID NO:4. Also preferably, the second bacterial leader sequence binds to an antibody that specifically binds to an amino acid sequence set forth in SEQ ID NO:4. Also preferably, the second bacterial leader sequence comprises the amino acid sequence set forth in SEQ ID NO:4.

[0053] The third peptidyl fragment can have an suitable length. For example, the third peptidyl fragment comprises about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, ,26, 27, 28, 29 or 30 amino acid residues. Preferably, the third peptidyl fragment comprises about 20 amino acid residues.

[0054] The isolated chimeric protein of can further comprise, at its C-terminus, a third peptidyl fragment comprising a peptide tag. Any suitable tag can be used. For example, the tag can be FLAG, HA, HA1, c-Myc, 6-His, AU1, EE, T7, 4A6, ε, B, gE and Ty1 tag (*See* Table 3).

Table 3. Exemplary epitope tag systems

Epitope	Peptide	SEQ ID	Antibody	Reference
FLAG	AspTyrLysAspAspAspLys	11	4E11	Prickett[1]
HA	TyrProTyrAspValPRoAspTyrAla	12	12Ca5	Xie[2]

Epitope	Peptide	SEQ ID	Antibody	Reference
HA1	CysGlnAspLeuProGlyAsnAspAsnSerThr	13	mouse MAb	Nagelkerken[3]
c-Myc	GluGlnLysLeuIleSerGluGluAspLeu	14	9E10	Xie[2]
6-His	HisHisHisHisHisHis	15	BAbCO*	
AU1	AspThrTyrArgTyrIle	16	BAbCO	
EE	GluTyrMetProMetGlu	17	anti-EE	Tolbert[4]
T7	AlaSerMetThrGlyGlyGlnGlnMetGlyArg	18	Invitrogen	Chen[5] Tseng[6]
4A6	SerPheProGlnPheLysProGlnGluIle	19	4A6	Rudiger[7]
ε	LysGlyPheSerTyrPheGlyGluAspLeuMetPro	20	anti-PKCε	Olah[8]
B	GlnTyrProAlaLeuThr	21	D11, F10	Wang[9]
gE	GlnArgGlnTyrGlyAspValPheLysGlyAsp	22	3B3	Grose[10]
Ty1	GluValHisThrAsnGlnAspProLeuAsp	23	BB2, TYG5	Bastin[11]

1. Prickett, *et al.*, *BioTechniques*, 7(6):580-584 (1989)
2. Xie, *et al.*, *Endocrinology*, 139(11):4563-4567 (1998)
3. Nagelkerke, *et al.*, *Electrophoresis*, 18:2694-2698 (1997)
4. Tolbert and Lameh, *J. Neurochem.*, 70:113-119 (1998)
5. Chen and Katz, *BioTechniques*, 25(1):22-24 (1998)
6. Tseng and Verma, *Gene*, 169:287-288 (1996)
7. Rudiger, *et al.*, *BioTechniques*, 23(1):96-97 (1997)
8. Olah, *et al.*, *Biochem.*, 221:94-102 (1994)
9. Wang, *et al.*, *Gene*, 169(1):53-58 (1996)
10. Grose, U.S. Patent No. 5,710,248
11. Bastin, *et al.*, *Mol. Biochem. Parasitology*, 77:235-239 (1996)

Invitrogen, Sigma, Santa Cruz Biotech

[0055] In an example, the isolated chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:5

(MGGSGDDDDLALAVTKSSSLLIVGAGTWGTSTALHLARRGYTNVTVLD
PYPVPSAISAGNDVNKVISSGQYSNNKDEIEVNEILAEEAFNGWKNDPLFK
PYYHDTGLLMSACSQEGLDRLGVRVRPGEDPNLVELTRPEQFRKLAPEGV
LQGDFPGWKGYFARSGAGWAHARNALVAAAREAQRMGVKFVTGTPQG
RVVTLIFENNDVKGAVTGDGKIWRAERTFLCAGASAGQFLDFKNQLRPT
AWTLVHIALKPEERALYKNIPVIFNIERGFFFEPDEERGEIKICDEHPGYTN
MVQSADGTMMSIPFEKTQIPKEAETRVRALLKETMPQLADRPFSFARICW
CADTANREFLIDRHPQYHSLVLGCGASGRGFKYLPSIGNLIVDAMEGKVP

QKIHELIKWNPDIAANRNWRDTLGRFGGPNRVMDFHDVKEWTNVQYRDI
SKLKGELEGLPIPNPLLRTGHHHHHH).

[0056] In another aspect, the present invention is directed to an isolated nucleic acid comprising a nucleotide sequence encoding a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase.

[0057] In one example, the isolated nucleic acid comprises a nucleotide sequence encoding the chimeric protein comprising the amino acid sequence set forth in SEQ ID NO:5. In another example, the isolated nucleic acid comprises a nucleotide sequence set forth in SEQ ID NO:6
(ATGGGAGGTTCGGGTGACGATGATGACCTGGCTCTCGCCGTCACTAA
GTCATCATCTCTCCTGATCGTTGGTGCCGGGACTTGGGGCACCTCAAC
GGCTCTGCACCTCGCGCGCCGCGGATATACCAACGTTACCGTGCTGGA
CCCCTATCCTGTCCCTAGCGCCATCTCCGCCGGAAACGACGTGAACAA
AGTCATTAGCAGTGGCCAATATTCGAATAACAAAGACGAAATCGAAG
TGAATGAGATCTTGGCGGAAGAGGCGTTTAACGGTTGGAAGAACGAC
CCGCTTTTCAAACCGTATTATCATGATACGGGCCTGCTGATGTCTGCTT
GCTCGCAGGAGGGCCTGGATCGCCTGGGCGTCCGGGTACGTCCGGGCG
AGGATCCTAATCTGGTGGAACTTACCCGCCCGGAGCAATTTCGTAAAC
TGGCCCCGGAAGGCGTGTTGCAAGGTGATTTTCCGGGTTGGAAAGGGT
ACTTTGCGCGTTCCGGCGCTGGCTGGGCACATGCAAGGAATGCCTTAG
TGGCAGCAGCACGCGAAGCACAGCGCATGGGTGTAAAATTTGTTACTG
GCACCCCGCAGGGTCGTGTAGTCACGTTAATCTTTGAAAATAACGATG
TAAAAGGTGCCGTTACGGGCGATGGCAAAATTTGGAGAGCGGAACGT
ACATTCCTGTGTGCTGGGGCTAGCGCGGGTCAGTTCCTAGATTTCAAG
AATCAACTTCGACCAACCGCTTGGACCCTGGTACACATTGCGTTAAAA
CCGGAAGAACGTGCGTTGTACAAAAATATACCGGTTATCTTTAACATC
GAACGGGGGTTTTTCTTTGAACCCGATGAGGAGCGCGGTGAGATTAAA
ATATGCGATGAACACCCGGGCTACACAAATATGGTCCAGAGTGCAGA
CGGCACGATGATGAGCATTCCGTTCGAAAAAACCCAGATTCCAAAAG

AAGCCGAAACGCGCGTTCGGGCCCTGCTGAAAGAGACAATGCCCCAG
CTGGCAGACCGTCCATTCAGCTTCGCACGCATTTGCTGGTGTGCCGAT
ACCGCGAATCGCGAATTCCTGATAGATCGACATCCGCAGTACCACAGT
CTTGTGTTGGGCTGTGGTGCGAGCGGAAGAGGGTTTAAATATCTGCCT
TCTATTGGGAATCTCATTGTTGACGCGATGGAAGGTAAAGTGCCGCAA
AAAATTCACGAATTAATCAAGTGGAACCCGGACATTGCGGCGAACCGT
AACTGGCGTGATACTCTGGGGCGTTTTGGCGGTCCAAATCGTGTGATG
GATTTTCATGATGTGAAGGAATGGACCAATGTTCAGTATCGTGATATT
TCCAAGCTGAAAGGAGAGTTGGAAGGTaaGCCAATCCCTAACCCGTTA
CTGCGCACAGGCCATCACCATCATCATCATTAA).

[0058] In still another example, the isolated nucleic acid comprising a nucleotide sequence complementary to the nucleotide sequence encoding a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase.

[0059] A recombinant cell containing the nucleic acid, or a complementary strand thereof, encoding a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase, is contemplated.

[0060] A method of producing a chimeric protein is also contemplated, which method comprising growing a recombinant cell containing the nucleic acid encoding a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase, such that the encoded chimeric protein is expressed by the cell, and recovering the expressed chimeric protein. The product of the method is further contemplated.

[0061] The chimeric proteins and the nucleic acids encoding the chimeric proteins can be prepared by any suitable methods, *e.g.*, chemical synthesis, recombinant production or a combination thereof (*See e.g.*, Current Protocols in Molecular Biology, Ausubel, et al. eds., John Wiley & Sons, Inc. (2000) and Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989)).

C. Methods and kits for assaying for a glycated protein using a chimeric protein

[0062] In still another aspect, the present invention is directed to a method for assaying for a glycated protein in a sample, which method comprises: a) contacting a sample to be assayed with a protease to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) contacting said generated glycated peptide or glycated amino acid with a chimeric protein comprising, from N-terminus to C-terminus: i) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and ii) a second peptidyl fragment comprising an amadoriase, to oxidize said glycated peptide or glycated amino acid; and c) assessing oxidation of said glycated peptide or glycated amino acid by said chimeric protein to determine the presence and/or amount of said glycated protein in said sample.

[0063] The present methods can be used to assay any suitable sample. Preferably, the sample is a blood sample, *e.g.*, a plasma, serum, red blood cell or whole blood sample.

[0064] The present methods can be used to assay any suitable glycated proteins. Preferably, the glycated protein to be assayed is glycoalbumin or glycohemoglobin.

[0065] Any suitable protease can be used in the present methods. Either an endo-type protease or an exo-type protease can be used. Exemplary endo-type proteases include trypsin, α-chymotrypsin, subtilisin, proteinase K, papain, cathepsin B, pepsin, thermolysin, protease XVII, protease XXI, lysyl-endopeptidase, prolether and bromelain F. Exemplary exo-type proteases include

an aminopeptidase or a carboxypeptidase. In one example, the protease is proteinase K, pronase E, ananine, thermolysin, subtilisin or cow pancreas proteases.

[0066] The protease can be used to generates a glycated peptide of any suitable size. For example, the protease can be used to generates a glycated peptide from about 2 to about 30 amino acid residues. In another example, the protease is used to generate glycated glycine, glycated valine or glycated lysine residue or a glycated peptide comprising glycated glycine, glycated valine or glycated lysine residue.

[0067] Any suitable chimeric proteins, including the ones described in the above Section B, can be used in the present methods. In one example, the chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:5. In another example, the chimeric protein is encoded by the nucleotide sequence set forth in SEQ ID NO:6.

[0068] The oxidation of the glycated peptide or glycated amino acid can be assessed by any suitable methods. For example, the oxidation of the glycated peptide or glycated amino acid can be assessed by assessing consumption of the glycated peptide or glycated amino acid, H_2O or O_2 in the oxidation reaction or the formation of the oxidized glucose (glucosone), H_2O_2 or the amino acid in the oxidation reaction.

[0069] The O_2 consumption by can be assessed by any suitable methods. For example, the O_2 consumption can be assessed by an oxygen electrode.

[0070] The H_2O_2 formation can be assessed by any suitable methods. For example, the H_2O_2 formation can be assessed by a peroxidase. Any peroxidase can be used in the present methods. More preferably, a horseradish peroxidase is used. For example, the horseradish peroxidases with the following GenBank accession Nos. can be used: E01651; D90116 (prxC3 gene); D90115 (prxC2 gene); J05552 (Synthetic isoenzyme C(HRP-C)); S14268 (neutral); OPRHC (C1 precursor); S00627 (C1C precursor); JH0150 (C3 precursor); S00626 (C1B precursor); JH0149 (C2 precursor); CAA00083 (Armoracia rusticana); and AAA72223 (synthetic horseradish peroxidase isoenzyme C (HRP-C)). In another

example, the H_2O_2 formation can be assessed by a peroxidase and Trinder reaction. The glycated peptide or glycated amino acid can be contacted with the chimeric protein and the peroxidase sequentially or simultaneously.

[0071] The glucosone formation can be assessed by any suitable methods. For example, the glucosone formation can be assessed by a glucose oxidase. Any suitable glucose oxidase can be used. For example, glucose oxidases encoded by the nucleotide sequences with the following GenBank accession Nos. can be used: AF012277 (Penicillium amagasakiense); U56240 (Talaromyces flavus); X16061 (Aspergillus niger gox gene); X56443 (A.niger god gene); J05242 (A.niger); AF012277 (Penicillium amagasakiense); U56240 (Talaromyces flavus); X16061 (Aspergillus niger gox gene); X56443 (A.niger god gene); J05242 (A.niger glucose). Preferably, the nucleotide sequences with the GenBank accession No. J05242 (*See also* Frederick, *et al.*, *J. Biol. Chem.*, 265(7):3793-802 (1990)) and the nucleotide sequences described in U.S. Patent No. 5,879,921 can be used in obtaining nucleic acid encoding glucose oxidase.

[0072] In another example, the glucosone formation can be assessed by a combination of glucose 6-phosphate dehydrogenase and hexokinase. Any suitable glucose 6-phosphate dehydrogenase can be used. For example, glucose 6-phosphate dehydrogenase disclosed in the following patents and patent applications can be used: WO 03/042389, WO 01/98472, WO 93/06125, and U.S. Patent Nos. 6,127,345, 6,069,297, 5,856,104, 5,308,770, 5,244,796, 5,229,286, 5,137,821 and 4,847,195. Any suitable hexokinase can be used. For example, hexokinase disclosed in the following patents and patent applications can be used: WO 02/20795, US2002/009779, WO 01/90378, WO 01/90325, WO 01/68694, WO 01/47968 and U.S. Patent No 5,948,665.

[0073] If desirable, the protease can be inactivated before or current with the contact between the glycated peptide or glycated amino acid and the chimeric protein. The protease can be inactivated by any suitable methods. For example, the protease can be inactivated by a heat treatment or an inhibitor of the protease.

[0074] If desirable, interference of the assay can be countered. For example, ascorbate interference can be countered using a copper (II) compound, a

cholic acid or a bathophenanthroline disulphonic acid or a mixture thereof. Bilirubin interference can be countered using a ferrocyanide salt.

[0075] The present methods can be used for any suitable purpose. Preferably, the method used in the prognosis or diagnosis of a disease or disorder, *e.g.*, diabetes.

[0076] In yet another aspect, the present invention is directed to a kit for assaying for a glycated protein in a sample, which kit comprises: a) a protease to generate glycated peptide or glycated amino acid from a glycated protein, if contained in a sample; b) a chimeric protein comprising, from N-terminus to C-terminus: i) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and ii) a second peptidyl fragment comprising an amadoriase, to oxidize said glycated peptide or glycated amino acid; and c) means for assessing oxidation of said glycated peptide or glycated amino acid by said chimeric protein to determine the presence and/or amount of said glycated protein in said sample.

[0077] Any suitable means can be included in the present kits. For example, the means for assessing oxidation of said glycated peptide or glycated amino acid by said chimeric protein can comprise a peroxidase. Preferably, the chimeric protein and the peroxidase are formulated in a single composition.

D. Methods and kits for assaying for a glycated protein using proteinase K and an amadoriase

[0078] In yet another aspect, the present invention is directed to a method for assaying for a glycated protein in a sample, which method comprises: a) contacting a sample to be assayed with a proteinase K to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) contacting said generated glycated peptide or glycated amino acid with an amadoriase to oxidize said glycated peptide or glycated amino acid; and c) assessing oxidation of said glycated peptide or glycated amino acid by said amadoriase to determine the presence and/or amount of said glycated protein in said sample.

[0079] The present methods can be used to assay any suitable sample. Preferably, the sample is a blood sample, *e.g.*, a plasma, serum, red blood cell or whole blood sample.

[0080] The present methods can be used to assay any suitable glycated proteins. Preferably, the glycated protein to be assayed is glycoalbumin or glycohemoglobin.

[0081] Any suitable amadoriase, including the ones described in the above Sections B and C, can be used in the present methods. For example, the amadoriase can comprise a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase. Preferably, the chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:5. Also preferably, the chimeric protein is encoded by the nucleotide sequence set forth in SEQ ID NO:6.

[0082] The oxidation of the glycated peptide or glycated amino acid can be assessed by any suitable methods. For example, the oxidation of the glycated peptide or glycated amino acid can be assessed by assessing consumption of the glycated peptide or glycated amino acid, H_2O or O_2 in the oxidation reaction or the formation of the oxidized glucose (glucosone), H_2O_2 or the amino acid in the oxidation reaction.

[0083] The O_2 consumption can be assessed by any suitable methods. For example, the O_2 consumption can be assessed by an oxygen electrode.

[0084] The H_2O_2 formation can be assessed by any suitable methods. For example, the H_2O_2 formation can be assessed by a peroxidase. Any peroxidase, including the ones described in the above Sections C, can be used in the present methods. More preferably, a horseradish peroxidase is used. In another example, the H_2O_2 formation can be assessed by a peroxidase and Trinder reaction. The glycated peptide or glycated amino acid can be contacted with the chimeric protein and the peroxidase sequentially or simultaneously.

[0085] The glucosone formation can be assessed by any suitable methods. For example, the glucosone formation can be assessed by a glucose oxidase. Any suitable glucose oxidase, including the ones described in the above Sections C, can be used.

[0086] In another example, the glucosone formation can be assessed by a combination of glucose 6-phosphate dehydrogenase and hexokinase. Any suitable glucose 6-phosphate dehydrogenase, including the ones described in the above Sections C, can be used. Any suitable hexokinase, including the ones described in the above Sections C, can be used.

[0087] Any suitable proteinase K can be used in the present methods. For example, proteinase K disclosed in the following patents and patent applications can be used: WO 02/072634, WO 02/064760, WO 96/28556, and U.S. Patent Nos. 6451574 and 5344770. Preferably, proteinase K from *Tritirachium album* is used (*See e.g.*, Sigma-Aldrich Catalog No. 82452).

[0088] If desirable, the proteinase K can be inactivated before or concurrent with the contact between the glycated peptide or glycated amino acid and the amadoriase. For example, the proteinase K can be inactivated by a heat treatment or an inhibitor of the proteinase K.

[0089] If desirable, interference of the assay can be countered. For example, ascorbate interference can be countered using a copper (II) compound, a cholic acid or a bathophenanthroline disulphonic acid or a mixture thereof. Bilirubin interference can be countered using a ferrocyanide salt.

[0090] The present methods can be used for any suitable purpose. Preferably, the method used in the prognosis or diagnosis of a disease or disorder, *e.g.*, diabetes.

[0091] In yet another aspect, the present invention is directed to a kit for assaying for a glycated protein in a sample, which kit comprises: a) a proteinase K to generate a glycated peptide or a glycated amino acid from a glycated protein, if contained in said sample; b) an amadoriase to oxidize said glycated peptide or glycated amino acid; and c) means for assessing oxidation of said glycated peptide

or glycated amino acid by said amadoriase to determine the presence and/or amount of said glycated protein in said sample.

[0092] Any suitable means can be included in the present kits. For example, the means for assessing oxidation of said glycated peptide or glycated amino acid by said chimeric protein can comprise a peroxidase. Preferably, the chimeric protein and the peroxidase are formulated in a single composition.

[0093] Any suitable amadoriase, including the ones described in the above Sections B and C, can be used in the present kits. For example, the amadoriase can comprise a chimeric protein, which chimeric protein comprises, from N-terminus to C-terminus: a) a first peptidyl fragment comprising a bacterial leader sequence from about 5 to about 30 amino acid residues; and b) a second peptidyl fragment comprising an amadoriase. Preferably, the chimeric protein comprises the amino acid sequence set forth in SEQ ID NO:5. Also preferably, the chimeric protein is encoded by the nucleotide sequence set forth in SEQ ID NO:6.

E. Examples

Example 1. Glycated Serum Protein Enzymatic Assay Kit

[0094] **Intended Use.** The exemplary assay kit is for determination of glycated serum proteins (fructosamine) in human serum. Fructosamine is formed due to a non-enzymatic Maillard reaction between glucose and amino acid residues of proteins. In diabetic patients, elevated blood glucose levels correlate with increased fructosamine formation. Fructosamine is a medium term indicator of diabetic control (2-3 weeks).

[0095] **Assay Principle.** The exemplary enzymatic assay for glycated serum proteins (GSP) uses Proteinase K to digest GSP into low molecular weight glycated protein fragments (GPF), and uses Diazyme's specific fructosaminase™, a microorganism originated amadoriase to catalyze the oxidative degradation of Amadori product GPF to yield PF or amino acids, glucosone and H_2O_2. The H_2O_2 released is measured by a colorimetric Trinder end-point reaction. The absorbance at 550 nm is proportional to the concentration of glycated serum proteins (GSP).

$$\text{GSP} \xrightarrow{\text{Proteinase K}} \text{Glycated protein fragments (GPF)}$$

$$\text{GPF} \xrightarrow{\text{Fructosaminase}} \text{PF or amino acids} + H_2O_2$$

$$H_2O_2 + \text{TOOS} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{Color} + H_2O$$

Table 4. Reagent Table

Reagent 1 (R1) Lyophilized 2 x 20 mL	Proteinase K, buffer
Reagent 2 (R2) Lyophilized 2 x 5mL	Fructosaminase™, HRP, buffer

[0096] **Test Samples.** Use fresh patient serum or EDTA treated plasma samples. Plasma should be separated from cells immediately after collection. Samples can be stored at 4^0C for 2 weeks or up to 4 weeks when frozen.

[0097] **Reconstitution.** One vial of Reagents 1 is reconstituted with 20 mL of distilled water. Mix gently by inversion and then allow to stand for a minimum of 10 min at room temperature before use. The reconstituted R1 is stable for 4 weeks at 4°C. One vial of Reagent 2 is reconstituted with 5 mL of distilled water. Mix gently by inversion and then allow to stand at room temperature for minimum of 10 min before use. The reconstituted R2 is stable for 6 weeks at 4°C.

[0098] **Assay Procedure**

1. Pre-warm reconstituted R1 and R2 at room temperature.
2. Instrumental parameters.

   Wavelength: 550nm; reference 700 nm

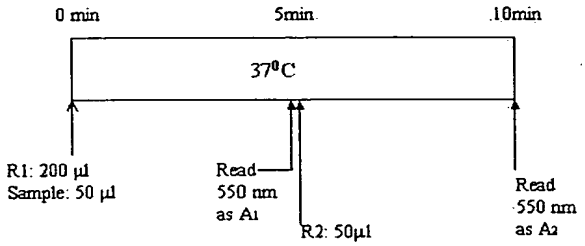
   Cuvette: 1 cm light path

Temperature: 37°C

3. Add 200 µL of the reconstituted R1 and 50 µL of sample or calibrator into cuvette. Mix and incubate for 5 min. Read absorbance at 550 nm as A_1.

4. Add 50 µL of the reconstituted R2, mix and incubate for further 5 min and then read the absorbance at 550 nm as A_2.

5. Reagent blank absorbance is read by using 50 µL of H_2O instead of sample or calibrator.



[0099] Calculation

$\Delta A = A_2 - A_1$

Concentration of glycated serum proteins (fructosamine) in sample:

$$\text{Fructosamine } (\mu\text{mole/L}) = \frac{\Delta A\text{sample} - \Delta A\text{blank}}{\Delta A\text{standard} - \Delta A\text{blankkk}} \text{ X Conc. of calibrator}$$

[0100] **Normal values.** Adults (20-60 years) have a normal range of 122-285µmol/L. Each laboratory should establish an expected range with a set of standards.

[0101] **Linearity and Sensitivity.** The assay is linear up to 1200 µmol/L and is sensitive at 30 µmol/L. The Diazyme Glycated Serum Protein (fructosamine) assay is precise with a mean inter-assay CV of <3% and mean intra assay CV of <2%. Assay data showed excellent correlation with the alternative fructosamine measurement method with $r^2 = 0.99$.

[0102] **Interferences.** The following analyte concentrations were not found to affect the assay:

Ascorbic acid (4 mg/dL)

Bilirubin (2 mg/dL)

Glucose (1200mg/dL

Hemoglobin (100mg/dL)

Triglycerides (250 mg/dL)

Uric acid (15 mg/dL)

[0103] **Calibration.** Fructosamine Calibrator (Cat. No. DZ112A-S) is required for calibration.

[0104] **Quality Control.** Fructosamine Controls (low and high) (Cat. No. DZ112A-C1 and DZ112A-C3) are recommended to use as control sera. One control (low or high) should be tested after every 30 samples. Values should fall within a specific range. If these values fall outside the range and repetition excludes error, the following steps should be taken:

1. Check instrument settings and light source;
2. Check reaction temperature;
3. Check expiry date of kit and contents; and
4. Check the quality of the water used for reagents reconstitution.

[0105] References

Armbuster DA, Fructosamine: Structure, Analysis and Clinical Usefulness. *Clin. Chem.* 1987; 33 (12): 2153-2163.

Kouzuma, T. *et al.* An enzymatic method for the measurement of glycated albumin in biological samples. *Clin. Chimi. Acta* 2002; 324: 61-71.

Example 2. Glycated Serum Protein Assay Precision and Linearity

METHOD COMPARISON

[0106] Determined by running 2 replicates of a set of random samples using both Diazyme GSP kit and Randox Fructosamine kit in one run. The analytical performance characteristics determined by Diazyme GSP kit were

comparable to those observed with Randox Fructosamine kit when assays were performed under the conditions as described in the Example 1 (*See also* Figure 1).

ASSAY LINEARITY

[0107] Determined by running 2 replicates of a set of series diluted serum samples in one run. The assay is linear from 40-856umole/L (*See* Figure 2).

INTERFERENCE

[0108] Determined by running 3 replicates each of a control sample in the absence and presence of various potential interference substances at indicated concentrations (See the following Table 5).

Table 5. Interference analysis

Interfering substances	Interfering substance concentration	% Interference
Ascorbic Acid	4 mg/dL	-0.3
Bilirubin	2 mg/dL	-0.6
Glucose	1200mg/dL	-0.6
Hemoglobin	100 mg/dL	-4.4
Triglycerol	250mg/dL	1.2
Uric Acid	15mg/dL	5.3

Example 3. Assay for Glycated Hemoglobin HbA1c

A. Glycated Valine Measurement:

[0109] 1. Mix 10ul 170mM Glycated Valine (G-Valine) (This value was assumed all valine was converted to G-Valine in the cooking procedure.) with 300ul R1 (80mMCHES, 30mmMOPS, 0.9%BRIJ). This mixture serves as sample stock solution (GVR1).

[0110] 2. Set spectrometer wavelength at 726nm, temperature at 37°C. Pipette 150ul R2 (30mMMES, 1mMCaCl2, 2mMWST-3, 1570U/ml Proteinase K) to a cuvette, add 0ul, 2.5ul, 5ul, 10ul, 15ul, 20ul above GVR1 respectively for dose response, make up the sample volume to 20 ul with H_2O in R1, incubate for 5min, get the first O.D. reading, then add 30ul R3 (0.08mMDA-64, 240mMTris,

180U/ml HRP, 20U/ml FAOD), incubate for 3 min, get the second O.D. reading. Calculate the O.D. difference between these two readings, using 20ul H_2O in R1 as control. Figures 3 and 4 show the dose-dependent reaction with fructosyl-valine.

B. Glycated Hemoglobin Measurement:

[0111] 1. Mix 10ul high level Glycated Hemoglobin (G-hg(HHg)), 10ul mid level G-hg(MHg), 10ul normal level G-hg (NMHg) respectively with 300ul R1.

[0112] 2. Set spectrometer wavelength at 570nm, temperature at 37°C. Pipette 150ul R2 to cuvette, add 20 ul above Hg in R1 as sample, read the O.D. after 4 min incubation, using H_2O in R1 as control. This O.D. reading gives the relative Hg concentration.

[0113] 3. Change the spectrometer wavelength to 726nm, get the first O.D. reading, add 30ul R3, incubate for 5min, get the second O.D. reading. Calculate the O.D. difference between these two readings. Normalize these O.D. differences of the three samples with their Hg concentrations. Figure 5 shows the dose-dependent signal with patient hemoglobin digested with a proteinase (5 min. digestion).

[0114] The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.